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FpvA-Mediated Ferric Pyoverdine Uptake in *Pseudomonas aeruginosa:* Identification of Aromatic Residues in FpvA Implicated in Ferric Pyoverdine Binding and Transport

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A number of aromatic residues were seen to cluster in the upper portion of the three-dimensional structure of the FpvA ferric pyoverdine receptor of *Pseudomonas aeruginosa*, reminiscent of the aromatic binding pocket for ferrichrome in the FhuA receptor of *Escherichia coli*. Alanine substitutions in three of these, W362, W391, and F795, markedly compromised ferric pyoverdine binding and transport, consistent with a role of FpvA in ferric pyoverdine recognition.

Iron acquisition by *Pseudomonas aeruginosa* is often facilitated by high-affinity iron chelating molecules, termed siderophores, that, together with cell surface receptors specific for the iron-siderophore complexes, serve to provide the organism with iron under nutritionally dilute conditions (20). A major siderophore produced by *P. aeruginosa* and, indeed, all fluorescent pseudomonads is pyoverdine, a mixed catecholate-hydroxamate siderophore characterized by a conserved dihydroxyquinoline chromophore to which is attached a peptide chain of variable length and composition (3, 18). This variation likely explains the noted specificity vis-à-vis pyoverdine utilization by *Pseudomonas* spp., where, for example, a given strain will often use only its own pyoverdine but not that of other *Pseudomonas* strains (4, 13), and suggests that the peptide moiety is involved in receptor recognition and binding. Some *P. aeruginosa* strains can also use so-called heterologous pyoverdines (i.e., those produced by other pseudomonads) of different chemical structure, though these often exhibit some peptide feature or partial amino acid sequence in common with the endogenous siderophore (1, 19, 28), again highlighting the importance of the peptide for receptor recognition. Three major, structurally distinct pyoverdines have been described for *P. aeruginosa*, dubbed types I, II, and III (17). Outer membrane receptors for all three have been described (FpvA [or FpvAI], FpvAII, and FpvAIII), and their genes have been cloned (7, 21). A second receptor for type I pyoverdine, FpvB, has also recently been reported for *P. aeruginosa* (10). The FpvA receptor, like other ferric siderophore receptors (12), has been shown to bind both iron-free and iron-bound siderophores (25–27), although there appear to be differences in the ways that iron-free and iron-bound pyoverdines interact with FpvA (5, 9). Still, both compete with a common or at least overlapping site on FpvA (5), and iron-bound pyoverdine effectively displaces iron-free pyoverdine on the receptor during transport (24, 25). Recently, the FpvA crystal structure with bound pyoverdine was solved at 3.6 Å (6), revealing a cluster of aromatic residues reminiscent of the FhuA ferrichrome receptor of *Escherichia coli*, where such residues were implicated in ferrichrome binding (8). We report here a study that confirms the importance of three residues in this cluster (W362, W391, and F795) in ferric pyoverdine binding and transport by FpvA.

Bacterial strains and plasmids used in this study are listed in Table 1. A pyoverdine-deficient Δ*pvdD* derivative of K1120 (an aminoglycoside-susceptible, *aphA* derivative of wild-type *P. aeruginosa* PAO1 strain K767) was constructed using plasmid pSUP202::Δ*pvd*. Briefly, pSUP202::Δ*pvd* was mobilized from *E. coli* S17-1 (29) into K1120 via conjugal transfer as described previously (30), and K1120 transconjugants carrying the plasmid in the chromosome were selected on 50 μg/ml tetracycline and 0.5 μg/ml imipenem (the latter to counterselect *E. coli* S17-1). To select for spontaneous loss of pSUP202 sequences, as a first step in selecting for strains in which the wild-type *pvdD* gene has been replaced by the deletion, three tetracycline- and imipenem-resistant colonies were individually inoculated into 5 ml Luria broth (L broth; Difco) and cultured overnight. These cultures were diluted 1:999 into fresh L broth (5 ml) and again cultured overnight. This was repeated daily over 8 days, after which dilutions of the cultures (10^{-5} to 10^{-7}) were plated onto L agar and colonies appearing after overnight incubation (800 were tested) were screened for loss of tetracycline resistance (on L agar supplemented with 100 μg/ml tetracycline). These tetracycline-sensitive, pSUP202-free isolates were then screened for an absence of fluorescence on iron-deficient succinate minimal agar plates, and one of these, K1203, was retained for further study. Iron-deficient succinate minimal medium has been described previously (16) and was supplemented with 0.05 % (wt/vol) Casamino Acids (CA). Cell envelopes were prepared as described previously (31) from *P. aeruginosa* strains cultured overnight in CA-supplemented iron-deficient succinate minimal medium and subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (10%

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
P. aeruginosa strains		
K767	PAO1 wild type	N. Gotoh, Kyoto, Japan
K1120	K767 *aphA*	22
K1203	K1120 Δ*pvdD*	This study
K2333	K1203 Δ*fpvA*	This study
K2334	K2333 *attB*::*fpvA* (WT)	This study
K2335	K2333 *attB*::*fpvA* (W362A)	This study
K2340	K2333 *attB*::*fpvA* (F366Y)[b]	This study
K2341	K2333 *attB*::*fpvA* (F369A)	This study
K2336	K2333 *attB*::*fpvA* (W391A)	This study
K2343	K2333 *attB*::*fpvA* (F795A)	This study
K2344	K2333 *attB*::*fpvA* (Y796A)	This study
K2345	K2333 *attB*::*fpvA* (Y801A)	This study
Plasmids[d]		
pSUP202Δ*pvdD*	pSUP202::Δ*pvdD*; Tc^r Ap^r Cm^r Tra^+	I. L. Lamont, University of Otago, New Zealand
mini-CTX1	*P. aeruginosa* chromosome integration vector; Tc^r	11
pCBS1	mini-CTX1 ΔBamHI-SacI[c]	This study
pCBS2	pCBS1::*fpvA* (WT)	This study
pFLP2	Carries gene for Flp recombinase; Ap^r Cb^r	11

[a] Amino acid changes in mutant FpvA proteins encoded by *fpvA* genes inserted into *P. aeruginosa* strain K2333 at the *attB* site are indicated in parentheses. WT, wild type.
[b] Mutation obtained via random mutagenesis of *fpvA*.
[c] mini-CTX1 derivative in which the BamHI and SacI sites have been engineered out of the vector.
[d] Plasmids mini-CTX1 and pCBS2 and their derivatives were maintained in *E. coli* and *P. aeruginosa* by the addition of tetracycline to the growth medium at 10 and 100 μg/ml, respectively. Plasmid pFLP2 was maintained in *E. coli* by the addition of ampicillin (100 μg/ml) and selected in *P. aeruginosa* by using carbenicillin (400 μg/ml). Plasmid pSUP202::Δ*pvdD* was maintained in *E. coli* by using 10 μg/ml tetracycline.

[wt/vol] acrylamide) (31) and Western immunoblotting (32) with an FpvA-specific rabbit polyclonal antiserum (21). To assess ^{59}Fe-pyoverdine binding and transport, *P. aeruginosa* cells were cultured for 24 h at 30°C in CA-supplemented iron-deficient succinate minimal medium, harvested by centrifugation (5 min at 13,000 rpm) in a microfuge, and washed with an equal volume of the same medium before being resuspended in one-half volume of this medium. One milliliter of washed cells was incubated on ice (binding assay) or with shaking at 37°C (transport assay) for 20 min with 50 μl ^{59}Fe-pyoverdine (14.5 nmol $^{59}FeCl_3$ [specific activity, 536 MBq/mg Fe; Amersham] diluted in 50 μl distilled H_2O containing 1 mM pyoverdine, incubated for 5 min at room temperature, and made up to 1 ml in CA-supplemented iron-deficient minimal medium) and subsequently harvested by centrifugation, washed twice with an equal volume of CA-supplemented iron-deficient succinate minimal medium, and resuspended in 1 ml of the same medium. ^{59}Fe bound to or transported by bacterial cells was measured with a scintillation counter.

Both a three-dimensional model (not shown) of FpvA based on the crystal structure of ferrichrome-bound FhuA (8) and the recently published FpvA crystal structure (6) reveal a cluster of aromatic residues (i.e., W362, F366, F369, W391, Y790, F795, Y796, and Y801) in the upper portion of the β-barrel region of FpvA, above the plane of the outer membrane (Fig. 1A). This is reminiscent of FhuA, where aromatic residues in the β-barrel of this receptor contribute to a high-affinity ferrichrome-binding site and an external aromatic pocket implicated in extracting ferrichrome from the external medium (8). However, only one of these, Y796, was implicated in pyoverdine binding in the FpvA crystal structure (6). Still, given possible differences in pyoverdine and ferric pyoverdine binding to FpvA (5, 9), these aromatic residues may be involved in binding ferric pyoverdine and not pyoverdine. Consistent with this, the above-highlighted aromatic cluster in FpvA was identified based on similarities to a cluster in FhuA implicated in binding that receptor's ferrated ligand, ferrichrome. To assess the involvement of these aromatic residues in FpvA function, then, alanine (and, in one instance, tyrosine) substitutions were engineered at each site and the impact on FpvA production and ferric pyoverdine binding and transport by *P. aeruginosa* expressing the mutant FpvA proteins was assessed. To engineer these substitutions, site-directed mutagenesis of *fpvA* on plasmid mini-CTX1 derivative pCBS2 was carried out using PCR with mutagenic primers, generally as described previously (23) (primer sequences and PCR parameters are available on request). Plasmid mini-CTX1 derivatives carrying wild-type or mutated *fpvA* were mobilized from *E. coli* DH5α (2) into the Δ*fpvA* Δ*pvdD P. aeruginosa* strain K2333 by using a previously described triparental mating procedure (32), with transconjugants carrying these plasmids in the chromosome (at the phage D113 *attB* site) selected on 70 μg/ml tetracycline and 25 μg/ml chloramphenicol, the latter to counterselect *E. coli*. The mini-CTX1 backbone was then excised from the chromosome, leaving behind the wild-type or mutant *fpvA* genes (i.e., producing FpvA with W362A, F366Y, F369A, W391A, Y790A, F795A, Y796A, or Y801A substitutions), by using the pFLP2-encoded Flp recombinase as described previously (11).

None of the substitutions adversely impacted FpvA production (Fig. 1C), although substitutions at W362 in particular and W391 and F795 to a substantial degree compromised ferric pyoverdine binding and transport (Table 2) (the Y790A substitution was not obtained). In a previous mutagenesis study, a peptide (18-mer) insertion at residue Y394 in FpvA (Y350 in

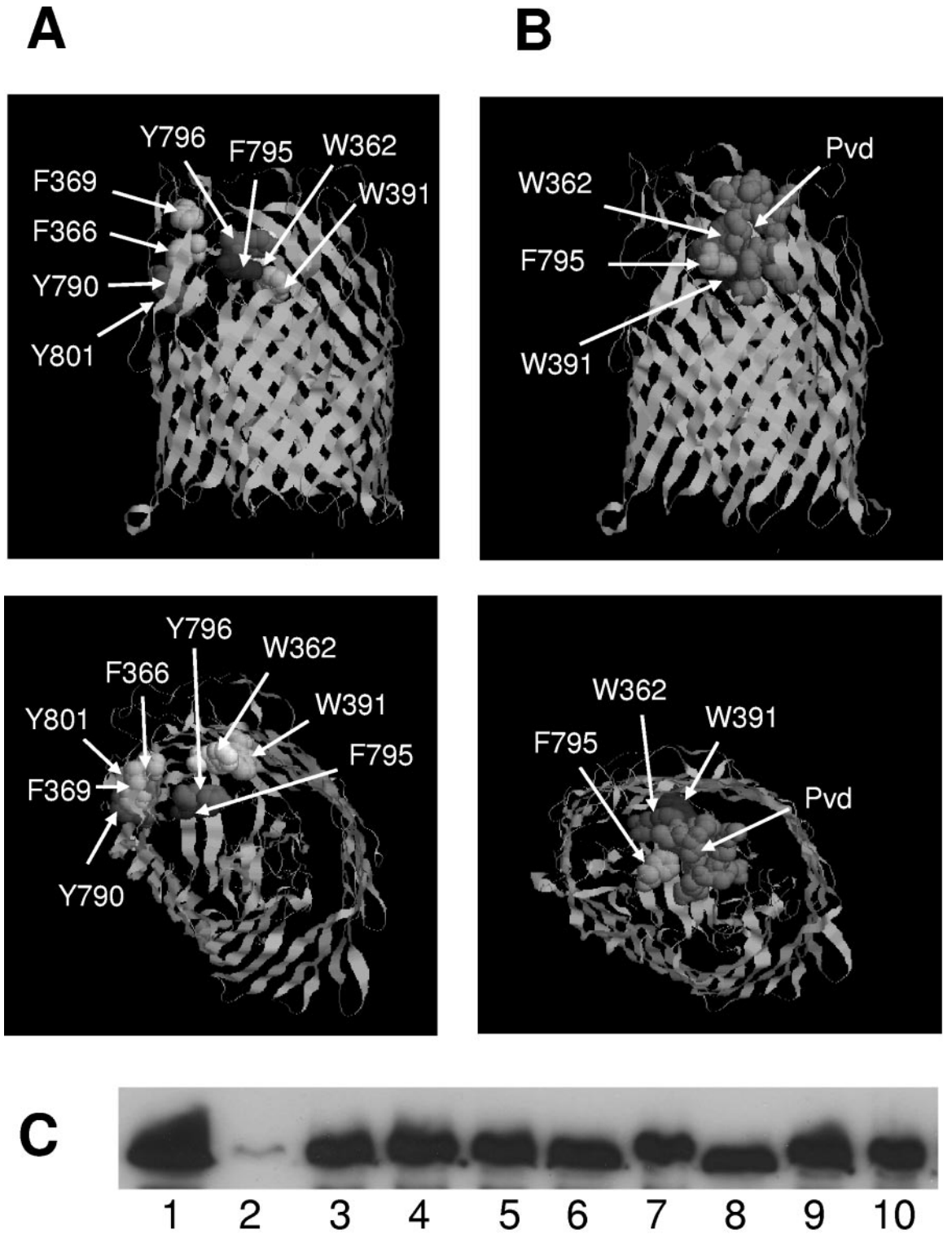

FIG. 1. Identification of aromatic residues in FpvA implicated in ferric pyoverdine binding and transport (A and B) and expression of FpvA proteins mutated in these residues (C). (A) Crystal structure of FpvA at 3.6 Å, taken from reference 6 (PDB code 1XKH), highlighting (in spacefill to assist visualization) the aromatic residues assessed for roles in ferric pyoverdine binding and transport. Top panel, side view; bottom panel, top view. (B) Crystal structure of FpvA, highlighting the aromatic residues confirmed to be involved in ferric pyoverdine binding and transport. The position of bound pyoverdine (Pvd) in the structure is also highlighted. Residues and pyoverdine are shown in spacefill to assist visualization. (C) Expression of FpvA proteins mutated at aromatic residues implicated in ferric pyoverdine binding and transport. Strains were cultured overnight in iron-deficient succinate minimal medium, and cell envelopes were prepared and immunoblotted using an FpvA-specific antiserum. Lane 1, K1203 (wild-type FpvA). Lanes 2 through 10, *P. aeruginosa* K2333 (K1203 Δ*fpvA*) expressing no FpvA (lane 2), wild-type FpvA (lane 3), $FpvA_{W362A}$ (lane 4), $FpvA_{F366Y}$ (lane 5), $FpvA_{F369A}$ (lane 6), $FpvA_{W391A}$ (lane 7), $FpvA_{F795A}$ (lane 8), $FpvA_{Y796A}$ (lane 9), and $FpvA_{Y801A}$ (lane 10).

TABLE 2. Pyoverdine-mediated iron binding and transport by *P. aeruginosa* expressing wild-type and mutant FpvA receptors[a]

FpvA protein produced[b]	Amt ^{59}Fe (cpm/A_{600} [%])[c]	
	Bound	Transported
WT (K1203)	1,183 (133)	7,191 (119)
WT	891 (100)	6,033 (100)
W362A	**149 (17)**	**1,290 (21)**
F366Y	918 (103)	7,874 (131)
F369A	716 (80)	7,656 (127)
W391A	**579 (65)**	**4,675 (77)**
F795A	**315 (35)**	**2,683 (44)**
Y796A	831 (93)	6,304 (104)
Y801A	696 (78)	7,397 (123)

[a] *P. aeruginosa* K2333 (Δ*pvdD* Δ*fpvA*) expressing mutant FpvA proteins from chromosome-integrated genes was cultured in CA-supplemented iron-deficient succinate minimal medium, incubated with ^{59}Fe-pyoverdine at 0°C (for binding assays) or 37°C (for transport assays), washed, and harvested on filters. Cell-bound ^{59}Fe was quantitated with a scintillation counter.

[b] The FpvA proteins carrying the indicated mutations were expressed from genes inserted into the chromosome of *P. aeruginosa* strain K2333 at the phage D113 *attB* site, with the exception of the wild-type (WT) FpvA protein produced by strain K1203, which carries the *fpvA* gene at its usual location.

[c] Values are reported as cpm and have been normalized to an A_{600} of 1.0. All values in column 2 have been adjusted for binding (152 cpm ^{59}Fe/A_{600}), and all values in column 3 have been adjusted for transport (2,840 cpm ^{59}Fe/A_{600}) by the FpvA$^-$ control strain K2333. Numbers in parentheses represent percent binding relative to the strain K2333 expressing wild-type FpvA. Values substantially below those for K2333 expressing wild-type FpvA are indicated in boldfaced type. Data are the means of two experiments.

that study, where residue numbering was based on the mature protein) also compromised FpvA-mediated ferric pyoverdine binding and transport (15), confirming the significance of this region (i.e., near W391) of the receptor in ferric pyoverdine recognition. Similarly, a peptide (8-mer) insertion at the G361 residue (G318 in the mature protein) adjacent to W362 was recently shown to obviate pyoverdine-mediated iron uptake (14). Interestingly, while residues W362, W391, and F795 were somewhat near the FpvA-bound pyoverdine in the crystal structure (W362, 3.07 Å; W391, 6.65, Å; F795, 3.97 Å) (Fig. 1B), they were not deemed sufficiently close to be implicated in pyoverdine binding (6). Still, it is interesting to note that W362 and W391, identified here as important for ferric pyoverdine binding, were implicated (6) as two of three tryptophan residues of FpvA responsible for fluorescence energy transfer (FRET) with the pyoverdine chromophore in earlier studies of FpvA-pyoverdine binding (25, 27). Moreover, these tryptophans also appear (6) to contribute to FRET in in vitro-reconstituted FpvA complexed with metal (i.e. gallium)-substituted pyoverdine (iron-bound pyoverdine is not fluorescent and, so, cannot be used in FRET assays) (9). Clearly, then, and in contrast to predictions based on the pyoverdine-FpvA crystal structure, W362 and W391 are important for ferric pyoverdine binding (and transport), suggesting that while pyoverdine and ferric pyoverdine may well bind to similar regions of the FpvA receptor (5), the specific details of binding differ and some differences exist with respect to residues involved in pyoverdine versus ferric pyoverdine binding. In further agreement with this, Y796 but not F795 was deemed sufficiently close to pyoverdine in the pyoverdine-bound FpvA structure to be a candidate residue for siderophore binding (6) and yet alanine substitutions at F795 but not Y796 compromised ferric pyoverdine binding and transport (Table 2). Thus, the pyoverdine-bound FpvA structure may not be particularly instructive with regard to the structural details of ferric pyoverdine binding or the identity of residues important for this binding.

Given the expected involvement of aromatic residues of FpvA in binding of the pyoverdine chromophore, it would be reasonable to assume that W362, W391, and F795 function in recognition of the dihydroxyquinoline moiety of iron-bound pyoverdine. Consistent with this, W391 is highly conserved in receptors for other pyoverdines (tryptophan in FpvAIII and FpvB and tyrosine in FpvAII) which, while differing in their peptide tails, share a conserved chromophore structure (3). Similarly, the conservation of an aromatic residue at positions equivalent to F795 in most of these other ferric pyoverdine receptors (phenylalanine in FpvB and tyrosine in FpvAIII) is also consistent with F795 of FpvA contributing to the recognition of the chromophore moiety of the bound ferric pyoverdine. Intriguingly, however, W362 (or any aromatic residue) is absent in receptors for type II and type III pyoverdines but is conserved in a second type I pyoverdine receptor broadly distributed in *P. aeruginosa*, FpvB (10). As such, this residue may be important for type I specificity in FpvA and explain, in part, the ability of FpvB to accommodate type I pyoverdine.

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